

Long-term release of antibiotics by carbon nanotube-coated titanium alloy 2 surfaces diminish biofilm formation by *Staphylococcus epidermidis*

Hirschfeld, Josefine; Akinoglu, Eser M; Wirtz, Dieter C; Hoerauf, Achim; Bekeredjian-Ding, Isabelle; Jepsen, Soren; Haddouti, El-Mustapha; Limmer, Andreas; Giersig, Michael

DOI:

[10.1016/j.nano.2017.01.002](https://doi.org/10.1016/j.nano.2017.01.002)

License:

Creative Commons: Attribution-NonCommercial-NoDerivs (CC BY-NC-ND)

Document Version

Peer reviewed version

Citation for published version (Harvard):

Hirschfeld, J, Akinoglu, EM, Wirtz, DC, Hoerauf, A, Bekeredjian-Ding, I, Jepsen, S, Haddouti, E-M, Limmer, A & Giersig, M 2017, 'Long-term release of antibiotics by carbon nanotube-coated titanium alloy 2 surfaces diminish biofilm formation by *Staphylococcus epidermidis*', *Nanomedicine: Nanotechnology, Biology and Medicine*, vol. 13, no. 4, pp. 1587–1593. <https://doi.org/10.1016/j.nano.2017.01.002>

[Link to publication on Research at Birmingham portal](#)

General rights

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

- Users may freely distribute the URL that is used to identify this publication.
- Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
- User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
- Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

Long-Term Release of Antibiotics by Carbon Nanotube-Coated Titanium Alloy Surfaces

Diminish Biofilm Formation by *Staphylococcus Epidermidis*

AUTHORS

Dr. Josefine Hirschfeld ^{‡,a,1}, Mr. Eser M. Akinoglu ^{‡,b,c}, Prof. Dr. Dieter C. Wirtz ^d, Prof. Dr. Achim Hoerauf ^e, PD Dr. Isabelle Bekeredjian-Ding ^f, Prof. Dr. Dr. Søren Jepsen ^a, Mr. El-Mustapha Haddouti ^d, Dr. Andreas Limmer ^{§,d}, Prof. Dr. Michael Giersig ^{§,b,g}

AUTHOR ADDRESSES

^a Department of Periodontology, Center of Dental and Oral Medicine, University Hospital Bonn, Welschnonnenstr. 17, 53111 Bonn, Germany†, E-mail: jepsen@uni-bonn.de

^b Department of Physics, Freie Universität Berlin, Arnimallee 14, 14195 Berlin, Germany
E-mail: giersig@physik.fu-berlin.de

^c Max Planck Institute of Colloids and Interfaces, Am Mühlenberg 1, 14476 Potsdam, Germany, E-mail: esera@zedat.fu-berlin.de

^d Department of Orthopedics, University Hospital Bonn, Sigmund-Freud-Str. 25, 53127 Bonn, Germany, E-mail: dieter.wirtz@ukb.uni-bonn.de; el-mustapha.haddouti@ukb.uni-bonn.de; andreas.limmer@uni-bonn.de

^e Institute of Medical Microbiology, Immunology and Parasitology, University Hospital Bonn, Sigmund-Freud-Str. 25, D-53127 Bonn, Germany, E-mail: achim.hoerauf@ukb.uni-bonn.de

^f Division of EU co-operation/Microbiology, Paul-Ehrlich Institut, Paul-Ehrlich-Str. 51-59, 63225 Langen, Germany, E-mail: isabelle.Bekeredjian-Ding@pei.de

^g Helmholtz-Zentrum Berlin für Materialien und Energie GmbH, Institute of Nanoarchitectures for Energy Conversion, Hahn-Meitner-Platz 1, 14109 Berlin, Germany

¹Present Address

Department of Restorative Dentistry, Birmingham Dental School and Hospital, University of Birmingham, 5 Mill Pool Way, Birmingham B5 7EG, UK

Corresponding Author

Josefine Hirschfeld, Department of Restorative Dentistry, Birmingham Dental School and Hospital, University of Birmingham, 5 Mill Pool Way, Birmingham B5 7EG, UK, E-mail: j.hirschfeld@bham.ac.uk, Phone: +44 121 4665496, Fax: +44 121 237 2809

Author Contributions

E.M.A. and M.G. designed and manufactured the MWCNT-covered TiAl6V4 titanium alloy surfaces. J.H. conducted the microbiological assays. M.G. and A.L. conceived the projects and designed the experiments. D.C.W. and M.H. provided input on implementing the experiments. A.H., I.B.-D. and S.J. contributed to the manuscript revision. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡, § These authors contributed equally.

Conflict of interest and financial disclosure

The authors declare that there is no conflict of interest regarding the publication of this paper. This work was supported by the German Society for Dental and Oral Medicine (DGZMK) and by the German Society of Periodontology (DGParo/2-RST-13).

Abstract word count: 150

Complete manuscript word count (including body text and figure legends, but excluding abstract, title page, and references): 3,757

Number of references: 61

Number of figures/tables: 2 figures, 1 graphical abstract

ABSTRACT

Bacterial biofilms cause a considerable amount of prosthetic joint infections every year, resulting in morbidity and expensive revision surgery. To address this problem, surface modifications of implant materials such as carbon nanotube (CNT) coatings have been investigated in the past years. CNTs are biologically compatible and can be utilized as drug delivery systems. In this study, multi-walled carbon nanotube (MWCNT) coated TiAl6V4 titanium alloy discs were fabricated and impregnated with Rifampicin, and tested for their ability to prevent biofilm formation over a period of ten days. Agar plate-based assays were employed to assess the antimicrobial activity of these surfaces against *Staphylococcus epidermidis*. It was shown that vertically aligned MWCNTs were more stable against attrition on rough surfaces than on polished TiAl6V4 surfaces. Discs with coated surfaces caused a significant inhibition of biofilm formation for up to five days. Therefore, MWCNT-modified surfaces may be effective against pathogenic biofilm formation on endoprostheses.

KEYWORDS

Multi-walled carbon nanotubes, drug delivery system, biofilm, *S. epidermidis*, antibiotics, prosthetic joint infection

ABBREVIATIONS

AB, antibiotic; PJI, prosthetic joint infection; CNT, carbon nanotube; MWCNT, multi-walled carbon nanotubes; PECVD, plasma enhanced chemical vapor deposition; VLS, vapor-liquid-solid

BACKGROUND

Bacteria commonly attach to natural and artificial surfaces within the host organism to form biofilms consisting of extracellular polysaccharides. Microbial adhesion to gut epithelium, teeth or skin is a physiological process that is strictly controlled by host defense mechanisms. These are, for example, epithelial shedding or bacterial killing by antimicrobial peptides, thus, preventing overgrowth or a shift towards pathogenicity (1, 2). Artificial surfaces such as prosthetic implants, however, are not well protected against colonization by biofilms and their overgrowth. One of the most frequently isolated bacteria from prosthetic devices is Gram-positive coagulase-negative *Staphylococcus epidermidis* (*S. epidermidis*), a prevalent microorganism inhabiting the skin and mucosal surfaces (3).

Bacteria gain access to prosthetic devices either during the surgery procedure, for instance after incomplete skin disinfection or via the blood stream, which they may enter through micro-injuries, a process termed bacteremia (4, 5). Importantly, microorganisms within biofilms are resistant to antibiotics (AB) and more difficult to eliminate (6). Prosthetic joint infection (PJI) is a relevant and serious complication of prosthetic joint implantation being associated with pain, loss of mobility and a mortality rate up to 2.5% (7). The relative incidence of PJI in the United States from 2001 to 2009 ranged between 2.0% and 2.4% of total hip arthroplasties and total knee arthroplasties. The annual cost of surgical revisions to US hospitals increased from \$320 million to \$566 million during this time range and was projected to exceed \$1.62 billion by 2020. As the demand for joint arthroplasty is expected to increase by up to 673% until 2030, the economic burden of PJI may equally increase (8, 9).

A possible approach to target PJI is the functional redesign of implant surfaces using nano technologies or antimicrobial coatings. Surfaces should simultaneously respond to various

biological and mechanical requirements and minimize bacterial adhesion and biofilm formation (10). In the past years, a range of nanocarriers has been proposed for the delivery of bioactive agents and for the inhibition of bacterial growth (11). Carbon nanotubes (CNT) were shown to be suitable structures for prolonged drug delivery, e.g. of anti-inflammatory drugs or growth factors (12, 13). They demonstrably can limit biofilm formation when anchored to a surface (14, 15), in suspension (16) or embedded in polymer nanocomposites (17). Furthermore, CNT structures were found to bind a range of antibiotics (18-22). Previously, our own experiments have shown that multi-walled carbon nanotubes (MWCNTs) are capable of stimulating the growth of stem cells and their differentiation into osteoblasts (23), whilst other groups have demonstrated an overall enhancement of osteoblast function by MWNT (24, 25). These effects were achieved by the MWCNTs alone without the addition of growth-promoting drugs.

Although AB have very limited effects against existing biofilms, they can successfully prevent their formation, when a continuous supply of AB is provided. Here, we present a method to reduce *in vitro* biofilm formation by *S. epidermidis* using AB impregnated MWCNT-modified TiAl6V4 titanium alloy surfaces. The novelty of this approach is the assessment of the liquid holding capacity and effectiveness against biofilm formation of these surfaces over time, thus providing further insight into medically relevant features of MWCNT-modified surfaces.

METHODS

MWCNT-coating of titanium alloy discs and scanning electron microscopy (SEM)

Vertically aligned MWCNTs were grown on roughened TiAl6V4 titanium alloy disc surfaces via plasma enhanced chemical vapor deposition (PECVD) (23). The roughness of the surfaces

is specified with $R_z = 10 \mu\text{m}$. Prior to the MWCNT growth, a 10 nm thin layer of nickel was deposited via electron beam evaporations that forms Ni droplets upon melting at the PECVD process temperature of approximately 750°C. These liquid Ni droplets act as catalysts in a tip growth type vapor-liquid-solid (VLS) mechanism where NH_3 and C_2H_2 are the gaseous precursors and the resulting vertically aligned MWCNTs the solid product. For details on the synthesis of MWCNTs via PECVD see reference (26). The majority of the utilized MWCNTs had an approximate length of 700 nm with closed tube ends encapsulating nickel catalyst particles. The tube diameters ranged from approximately 10 nm to 200 nm and inter-MWCNT distances were in the same range.

Bacterial culture

As a model bacterium and biofilm forming microbe, *S. epidermidis* (ATCC 35984) was used in this study. *S. epidermidis* was maintained on tryptic soy agar (TSA) (BD, Heidelberg, Germany). Before each experiment, overnight cultures were prepared in tryptic soy broth (TSB) (BD, Heidelberg, Germany) at 37°C on an orbital shaker. Next, the overnight culture was diluted 100x and grown to late logarithmic phase as monitored by optical density measurement ($\text{OD}_{600}=0.9\text{-}1.0$).

Preparation of titanium alloy discs

As a rule, discs were handled using forceps to avoid damages of the MWCNT surface. Discs were immersed in 10 $\mu\text{g/mL}$ Rifampicin (Sigma Aldrich, Seelze, Germany), which was assessed as the minimal inhibitory concentration (MIC), for 4 h at 4°C. Moreover, MIC_{50} , at which 50% of bacteria are inhibited, was measured at 5 $\mu\text{g/mL}$. No bacterial inhibition was observed at 1 $\mu\text{g/mL}$. Rifampicin was shown to be effective against *Staphylococcus* species (27). Next, discs were washed in sterile PBS for 30 s using a squirt bottle in order to fully remove excess Rifampicin solution. Immediately after washing and shaking off excess liquid,

the discs were placed into 1 mL of sterile PBS in 24-well plates for 1, 3, 5 or 10 days and kept at 4°C. By these means, the AB diffused into the PBS for various amounts of time (AB diffusion time) and the retention time for the liquid containing the AB could be assessed. As controls, rough discs without MWCNT modification as well as MWCNT discs were prepared in the same manner. From the geometric dimensions of the MWCNT coating the loading of the Rifampicin samples can be approximated to 250 pg/disc. This was calculated using the following parameters: MWCNT had an approximate length of 700 nm, closed tube ends and an average tube diameter and inter-MWCNT distance of 10 nm to 200 nm (arithmetic average=105 nm). Each disc had a MWCNT-coated surface of 60 mm², where approximately 4.5x10⁷ nanotubes were present per mm². The total volume of MWCNTs (0.018 mm³) was subtracted from the total volume of the surface (0.042 mm³). Therefore, the average volume of all MWCNT inter-spaces was 0.024 mm³ and the approximate liquid absorption capacity 25 nL/disc.

Assay procedure

After the appropriate AB diffusion time, excess PBS was shaken off the discs. They were then placed into 1 mL of fresh TSB containing a total of 1-2 x 10⁵ bacteria and incubated for 24 h at 37°C. The next day, each disc was removed from its well and washed three times in sterile PBS. The back surface of each disc was carefully wiped using a sterile ethanol-wetted cotton swab in order to only assess the biofilm that had grown on the surface of the discs.

Subsequently, discs were placed into 0.5 mL of sterile PBS within 48-well plates. By vigorously pipetting up and down for 1 min biofilms were removed from the discs. The suspensions were either measured for optical density at 490 nm (OD₄₉₀) or diluted 50,000 times, plated on TSA in triplicate and incubated at 37°C. Colony forming unites (CFU) were counted the following day. After detaching the biofilms, each disc was stained with a crystal violet solution to visually confirm that no bacteria were present on the disc surfaces. Our own

previously performed experiments along with a published method conducted under similar conditions (28) had shown that this approach was suitable for developing an appropriate washing protocol and to verify the absence of remnant bacteria on the discs. In these previous experiments, after crystal violet staining, the discs were further washed in ethanol over 24h, which led to detachment of remaining bacteria. OD₄₉₀ reading values were obtained and compared with negative controls.

In another experimental setup, the discs were placed upside-down on TSA inoculated with *S. epidermidis* in order to assess inhibition zones after 24 h of incubation at 37°C. In order to reveal possible differences between this method and the widely used method of agar diffusion using filter paper discs, both were compared regarding the diameter of inhibition zones. This was accomplished by using filter paper discs of the same size as the titanium discs. Rough and MWCNT-coated discs as well as filter paper discs were treated with 250 pg of Rifampicin and placed onto agar immediately, and inhibition zones were assessed after 24h.

Statistical analysis

Two-sided unpaired Student's *t*-test was applied to calculate significant differences between the discs in terms of biofilm formation and inhibition zones.

RESULTS

A major problem of current anti-microbial coatings (e.g. silver) is the massive cytotoxic effect, which impairs biofilm formation but also prevents bone regeneration (29). Therefore, anti-microbial nanostructures have to be capable of preventing biofilm formation for prolonged periods of time. At the same time, they must allow for, or even stimulate, differentiation of

stem cells into bone. Based on our previous work showing that MWCNT are ideal substrates for differentiation of osteoblasts from mesenchymal stem cells (23), we aimed to combine these characteristics with anti-biofilm properties, as a result of the prolonged release of AB.

CNT-coating of titanium alloy discs

For this study, MWCNTs with the length of 700 nm were used throughout all samples to ensure comparability. Scanning electron microscopy images of the multi-walled carbon nanotube on the sample discs are shown in **Figure 1**. A low magnification image of the rough TiAl6V4 titanium alloy disc covered homogenously with the MWCNTs is shown in **Figure 1A**. A higher magnification of the area encircled in a red square is shown in **Figure 1B** in top view and **Figure 1C** in 45° tilt perspective, respectively. The individual dots in **Figure 1B** correspond to MWCNTs where their diameter ranges from about 10-200 nm. The projection of the MWCNTs into this 45° image plane shows the length of the MWCNTs which is approximately 700 nm where few individual MWCNTs are longer. The darker shadows on the tips of the MWCNTs correspond to the encapsulated nickel particles from which the MWCNTs grew during the VLS mechanism during the PECVD growth. It is apparent, that the TiAl6V4 titanium alloy surface is roughened on a scale much larger than the dimensions of the MWCNTs. This attribute showed beneficial properties: a better stability of MWCNTs was observed on roughened compared to polished TiAl6V4 titanium alloy surfaces against shear forces that may occur during handling and sterilization of the discs. This is of significant importance when such coatings are to be applied to implants that are subject to surgery and the thereby accompanied handling.

Anti-biofilm and antibacterial properties

Interestingly, CNT by themselves were shown to be toxic towards several bacteria and can inhibit biofilm formation. Nevertheless, bacteria can quickly adapt to this challenge by

modifying their cell walls (30-32), requiring further efforts to create antibacterial surfaces. MWCNT-coated discs impregnated with quantities of Rifampicin as low as < 250 pg/disc, showed significant anti-biofilm effects. After a diffusion time of 1 d, biofilm growth was completely inhibited compared to rough discs pretreated with the AB. After 5 d, there was still a reduction by half, whereas at day 10, biofilm levels were equal to controls (rough discs), suggesting that Rifampicin had fully diffused into the buffer. Rough discs were able to retain the AB for only 1 d as assessed by comparison against controls without AB (**Figure 2A**). These results could be confirmed by optical density measurements of detached biofilm suspensions (**Figure 2B**). Moreover, visible inhibition zones occurred at 1 – 5 d of diffusion time (**Figure 2C**). After a diffusion time of 5 d, the inhibition zone was half in diameter compared to 1 and 3 d, implying that the concentration of Rifampicin had decreased to $5 \mu\text{g/mL}$ (MIC_{50}), and to $\leq 1 \mu\text{g/mL}$ after 10 d. No differences were seen in the antimicrobial effects of titanium alloy and filter paper discs when placed onto agar immediately after AB impregnation.

DISCUSSION

CNTs and materials based on nanotubes have several potential applications in medicine and biomedicine. They can act as growth substrates, tissue scaffolds or as carriers for various therapeutic and diagnostic agents (33-35). In the literature, loading of CNT and other nanotubular structures with osteogenic substances and growth factors has been described (36-38). Data on loading of CNTs with AB for prolonged drug delivery in this context, however, is limited (39, 40). The present study is, to our knowledge, the first to demonstrate biofilm inhibition over more than 5 days, indicating that vertically aligned MWCNT-structures can

serve as a reservoir for extended periods of time. The duration of AB delivery is crucial, as a considerable amount of bacterial PJI occur within the first months after surgery (41). Bacterial adherence to implanted prosthetic devices and subsequent pathologic inflammation can hinder initial implant healing and result in morbidity and costly revision surgery. Most advantageously, local delivery of AB can yield higher concentrations at the site of bacterial adhesion compared to systemic administration. Thus, common side effects such as impairment of the digestive system can be minimized (42). In the present study, the effect of MWCNT-coating on prolongation of AB release was assessed and quantified.

Interestingly, Malek *et al.* demonstrated the inhibition of biofilm growth by MWCNT alone, anchored to a silicon surface (15). The authors propose that the anti-biofilm effect was based on slight oscillations of the thin (6–20 nm) and less rigid MWCNT, preventing bacterial settlement on this unstable substrate. The diameters of the MWCNT used in our study was at 10–200 nm, leading to a higher surface rigidity and therefore providing an explanation for the undisturbed biofilm formation on control discs. Stability of surface coatings is of major importance, as they are subject to shear stress during surgery and wear (43, 44). Even though MWCNTs themselves are robust, their adhesion forces onto a substrate are orders of magnitude lower than macroscopic mechanical forces during handling, which is especially true for vertically aligned MWCNTs that are only attached with one of their tips to the support material. This obstacle can be overcome utilizing roughened surfaces, where the majority of the surface area is protected against shear forces as the peaks of the surface shadow the valleys of the morphology. A desirable side effect is, that the total surface area is increased compared to flat surfaces and the fact that implant materials are commonly designed to be roughened makes this approach very appealing.

Another way to design surfaces that allow for storage of a bioactive agent is the formation of nanopores. There is a large multitude of drug release studies using nanoporous aluminium oxide templates (Al_2O_3) (45). These were shown to be successful in delivering bioactive agents over prolonged periods of time. TiAl6V4 titanium alloy, however, is currently the material of choice for implantable devices, due to its better biocompatibility and bone-conductivity (46). Only few studies have assessed the release of drugs from nanoporous surfaces based on titanium. Ayon *et al.* demonstrated release of dexamethasone from 5 nm wide pores on titanium dioxide (TiO_2) films, but no time-dependent release was assessed in this study (47). Similarly, Ketabchi *et al.* monitored the release of albumin and vancomycin from nanoporous titanium surfaces, but this release was not evaluated over time (48). Lopez *et al.* showed that a porous TiO_2 -silica xerogel was able to release an anticonvulsant drug over a period of 500 h and that a slower release rate occurred at higher loading concentrations (49). The formation of nanopores on TiAl6V4 titanium alloys may be a promising approach for constructing antimicrobial implant surfaces. As these pores can be designed to have similar dimensions as the inter-tubular spaces in our study, they could also feature long-term liquid holding capacities. Moreover, in contrast to nanotubes, a nanoporous surface does not depend on adhesion onto a substrate and may therefore be mechanically more stable.

In the present study, our goal was to explore the possibility of MWCNT loading by capillary forces and to assess the liquid retention over time thereafter. Therefore, the discs were not dried after AB impregnation and washing. Regarding the possibility of future *in vivo* application, it is desirable to coat MWCNT surfaces of implanted devices immediately before implantation, according to the patient's medical needs. This can potentially be realized by immersing the MWCNT textured devices into a liquid carrier substance containing AB for drug loading. Importantly, dissolved AB may be more effective than when in a dried state (50), as they do not depend on liquid such as body fluids entering the nanotubular structures

to solubilize the AB. An improvement of our findings may be obtained by optimizing the geometric dimensions of the utilized MWCNT array to maximize the liquid loading volume. By increasing the AB concentration, the inhibition of biofilm formation may be further prolonged, however, our research was aimed at assessing the long-term effect of the specific MIC measured after 1 d. Another approach may be the utilization of MWCNTs with open ends to additionally store liquid media within a hollow tube, which is a common method to load CNT with low-density liquids (51). Moreover, a vacuum-assisted loading of the surfaces with a liquid of higher density/viscosity could be applied in order to decelerate substance release (52, 53). Notably, the surfaces roughness in our study was at $R_z = 10\text{-}12\text{ }\mu\text{m}$, whereas commercially available implants feature a roughness of approximately $R_z = 50\text{ }\mu\text{m}$ (54), indicating that an approximation of this value could further enhance AB holding and long-term release capacity.

A desirable goal of this translational approach is a slow release of AB from the implant surface within the vulnerable timeframe of initial healing. In the present study, very low concentrations of Rifampicin caused notable inhibition zones and inhibition of biofilm formation even after 5 days. In our experimental setup, discs were placed into physiological buffer for one to several days in order to assess the ability of MWCNT surfaces to retain the AB against efflux and diffusion. Significant and long-lasting bactericidal effects could be achieved by immersing the discs in Rifampicin solution for 4h. Thus, it can be concluded that short-term capillary forces can be applied to load MWCNT-modified surfaces where the liquid is stored in the volume between MWCNTs. Hence, loading and retention should be strongly dependent on the average MWCNT length, widths and inter-MWCNT distance and was approximated to 250 pg Rifampicin per sample disc. The diffusion of the Rifampicin can be assumed to follow one-dimensional Fick's law as a consequence of the coating geometry.

Similar diffusion rates may occur *in vivo*, therefore, MWCNT structures on an implanted device could be a successful vehicle for such long-term delivery of antimicrobial agents. Successful osseointegration is a crucial process after prosthetic joint implantation. The fact that osteoblasts readily grow on nanotube structures renders CNTs a suitable biomaterial coating (23, 55, 56). At the same time, it is undesirable that bacterial cells attach to CNTs more than to uncoated surfaces. Our experiments showed that MWCNT-coated TiAl6V4 titanium alloy discs did not lead to increased bacterial adhesion compared to non-coated discs, confirming previous findings (57) and reinforcing MWCNTs as a suitable surface modification. MWCNTs are mechanically robust and chemically inert, making them very promising tools for biological surface engineering. Good biocompatibility with host cells has been reported for MWCNTs with > 200 nm in length and > 20 nm in diameter (58).

Importantly, the approach of coating TiAl6V4 titanium alloy surfaces with CNTs can be very helpful in the context of individualized medicine. Here, microbial resistance profiles as well as AB allergies of susceptible individuals can be taken into account and the implanted device may be loaded with the appropriate antimicrobial agent prior to surgery. Preliminary experiments have been conducted by our group previously, using the biofilm-forming strains *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Staphylococcus aureus*. MWCNT-coated discs impregnated with the appropriate antibiotic led to results similar to those shown in the present study regarding inhibition biofilm formation over time. Therefore, we believe that such MWCNT surfaces treated with antibiotics could be applied in various bacterial infections and using different antibiotics. Future work should be implemented to optimize the MWCNT coatings towards a maximum loading capacity and optimal drug release properties. Subsequently, animal studies should be conducted in order to confirm the *in vitro* effects of prolonged biofilm inhibition found in this study under *in vivo* conditions. Also, it is important to assess their biological degradation and possible cytotoxic

effects (59-61). Advancing research of nanostructured surfaces as antimicrobial drug delivery systems may be an important step towards decreasing PJI events in the coming decades.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Klaus Ellmer from the Helmholtz Zentrum Berlin and Prof. Dr. James Deschner from the Center of Dental and Oral Medicine in Bonn for their valuable support throughout this study.

REFERENCES

1. Hänsch GM. Host Defence against Bacterial Biofilms: “Mission Impossible”? *ISRN Immunology*. 2012;**2012**:1.
2. Ribet D, Cossart P. How bacterial pathogens colonize their hosts and invade deeper tissues. *Microbes Infect*. 2015;**17**(3):173-83.
3. O’Gara JP, Humphreys H. Staphylococcus epidermidis biofilms: importance and implications. *J Med Microbiol*. 2001;**50**(7):582-7.
4. Tande AJ, Patel R. Prosthetic joint infection. *Clin Microbiol Rev*. 2014;**27**(2):302-45.
5. Hirschfeld J, Kawai T. Oral inflammation and bacteremia: implications for chronic and acute systemic diseases involving major organs. *Cardiovasc Hematol Disord Drug Targets*. 2015;**15**(1):70-84.
6. Donlan RM. Biofilms and device-associated infections. *Emerg Infect Dis*. 2001;**7**(2):277-81.
7. Lentino JR. Prosthetic joint infections: bane of orthopedists, challenge for infectious disease specialists. *Clin Infect Dis*. 2003;**36**(9):1157-61.

8. Kurtz SM, Lau E, Watson H, Schmier JK, Parvizi J. Economic burden of periprosthetic joint infection in the United States. *J Arthroplasty*. 2012;**27(8 Suppl)**:61-5.e1.
9. Kurtz S, Ong K, Lau E, Mowat F, Halpern M. Projections of primary and revision hip and knee arthroplasty in the United States from 2005 to 2030. *J Bone Joint Surg Am*. 2007;**89(4)**:780-5.
10. Gallo J, Holinka M, Moucha CS. Antibacterial surface treatment for orthopaedic implants. *Int J Mol Sci*. 2014;**15(8)**:13849-80.
11. Taylor E, Webster TJ. Reducing infections through nanotechnology and nanoparticles. *Int J Nanomedicine*. 2011;**6**:1463-73.
12. Luo X, Matranga C, Tan S, Alba N, Cui XT. Carbon nanotube nanoreservoir for controlled release of anti-inflammatory dexamethasone. *Biomaterials*. 2011;**32(26)**:6316-23.
13. Liu Z, Feng X, Wang H, Ma J, Liu W, Cui D, et al. Carbon nanotubes as VEGF carriers to improve the early vascularization of porcine small intestinal submucosa in abdominal wall defect repair. *Int J Nanomedicine*. 2014;**9**:1275-86.
14. Puckett SD, Taylor E, Raimondo T, Webster TJ. The relationship between the nanostructure of titanium surfaces and bacterial attachment. *Biomaterials*. 2010;**31(4)**:706-13.
15. Malek I, Schaber CF, Heinlein T, Schneider JJ, Gorb SN, Schmitz RA. Vertically aligned multi walled carbon nanotubes prevent biofilm formation of medically relevant bacteria. *J Mater Chem B*. 2016;**4(31)**:5228-35.
16. Dong X, Yang L. Inhibitory effects of single-walled carbon nanotubes on biofilm formation from *Bacillus anthracis* spores. *Biofouling*. 2014;**30(10)**:1165-74.
17. Goodwin DG, Xia Z, Gordon TB, Gao C, Bouwer EJ, Fairbrother DH. Biofilm development on carbon nanotube/polymer nanocomposites. *Environ Sci Nano*. 2016;**3(3)**:545-58.
18. Ji L, Chen W, Zheng S, Xu Z, Zhu D. Adsorption of sulfonamide antibiotics to multiwalled carbon nanotubes. *Langmuir*. 2009;**25(19)**:11608-13.

19. Ji L, Chen W, Duan L, Zhu D. Mechanisms for strong adsorption of tetracycline to carbon nanotubes: a comparative study using activated carbon and graphite as adsorbents. *Environ Sci Technol*. 2009;**43**(7):2322-7.
20. Tian Y, Gao B, Chen H, Wang Y, Li H. Interactions between carbon nanotubes and sulfonamide antibiotics in aqueous solutions under various physicochemical conditions. *J Environ Sci Health A Tox Hazard Subst Environ Eng*. 2013;**48**(9):1136-44.
21. Li H, Zhang D, Han X, Xing B. Adsorption of antibiotic ciprofloxacin on carbon nanotubes: pH dependence and thermodynamics. *Chemosphere*. 2014;**95**:150-5.
22. Cong Q, Yuan X, Qu J. A review on the removal of antibiotics by carbon nanotubes. *Water Sci Technol*. 2013;**68**(8):1679-87.
23. Giannona S, Firkowska I, Rojas-Chapana J, Giersig M. Vertically aligned carbon nanotubes as cytocompatible material for enhanced adhesion and proliferation of osteoblast-like cells. *J Nanosci Nanotechnol*. 2007;**7**(4-5):1679-83.
24. Sirinrath S, Chang Y, Xingcheng X, Brian WS, Thomas JW. Greater osteoblast functions on multiwalled carbon nanotubes grown from anodized nanotubular titanium for orthopedic applications. *Nanotechnology*. 2007;**18**(36):365102.
25. Subramani K, Pandravadana SN, Puleo DA, Hartsfield JK, Huja SS. In vitro evaluation of osteoblast responses to carbon nanotube-coated titanium surfaces. *Prog Orthod*. 2016;**17**:23.
26. Zhifeng R, Yucheng L, Wang Y. *Aligned Carbon Nanotubes - Physics, Concepts, Fabrication and Devices*. Berlin, Heidelberg: Springer; 2013, p. 300.
27. Aboltins CA, Page MA, Buising KL, Jenney AW, Daffy JR, Choong PF, et al. Treatment of staphylococcal prosthetic joint infections with debridement, prosthesis retention and oral rifampicin and fusidic acid. *Clin Microbiol Infect*. 2007;**13**(6):586-91.
28. Antoci V, Adams CS, Parvizi J, Davidson HM, Composto RJ, Freeman TA, et al. The inhibition of staphylococcus epidermidis biofilm formation by vancomycin-modified titanium

alloy and implications for the treatment of periprosthetic infection. *Biomaterials*.

2008;**29(35)**:4684-90.

29. Albers CE, Hofstetter W, Siebenrock KA, Landmann R, Klenke FM. In vitro cytotoxicity of silver nanoparticles on osteoblasts and osteoclasts at antibacterial concentrations. *Nanotoxicology*. 2013;**7(1)**:30-6.

30. Rodrigues DF, Elimelech M. Toxic effects of single-walled carbon nanotubes in the development of E. coli biofilm. *Environ Sci Technol*. 2010;**44(12)**:4583-9.

31. Kang S, Mauter MS, Elimelech M. Physicochemical determinants of multiwalled carbon nanotube bacterial cytotoxicity. *Environ Sci Technol*. 2008;**42(19)**:7528-34.

32. Zhu B, Xia X, Xia N, Zhang S, Guo X. Modification of Fatty Acids in Membranes of Bacteria: Implication for an Adaptive Mechanism to the Toxicity of Carbon Nanotubes. *Environ Sci Technol*. 2014;**48(7)**:4086-95.

33. Lee W, Parpura V. Chapter 6 - Carbon nanotubes as substrates/scaffolds for neural cell growth. *Prog Brain Res*. 2009;**180**:110-25.

34. Hu Y, Cai K, Luo Z, Xu D, Xie D, Huang Y, et al. TiO₂ nanotubes as drug nanoreservoirs for the regulation of mobility and differentiation of mesenchymal stem cells. *Acta Biomater*. 2012;**8(1)**:439-48.

35. Martincic M, Tobias G. Filled carbon nanotubes in biomedical imaging and drug delivery. *Expert Opin Drug Deliv*. 2015;**12(4)**:563-81.

36. Han ZJ, Ostrikov KK, Tan CM, Tay BK, Peel SA. Effect of hydrophilicity of carbon nanotube arrays on the release rate and activity of recombinant human bone morphogenetic protein-2. *Nanotechnology*. 2011;**22(29)**:295712.

37. Bae IH, Yun KD, Kim HS, Jeong BC, Lim HP, Park SW, et al. Anodic oxidized nanotubular titanium implants enhance bone morphogenetic protein-2 delivery. *J Biomed Mater Res B Appl Biomater*. 2010;**93(2)**:484-91.

38. Hirata E, Menard-Moyon C, Venturelli E, Takita H, Watari F, Bianco A, et al. Carbon nanotubes functionalized with fibroblast growth factor accelerate proliferation of bone marrow-derived stromal cells and bone formation. *Nanotechnology*. 2013;**24(43)**:435101.
39. Popat KC, Eltgroth M, Latempa TJ, Grimes CA, Desai TA. Decreased Staphylococcus epidermis adhesion and increased osteoblast functionality on antibiotic-loaded titania nanotubes. *Biomaterials*. 2007;**28(32)**:4880-8.
40. Lin WT, Tan HL, Duan ZL, Yue B, Ma R, He G, et al. Inhibited bacterial biofilm formation and improved osteogenic activity on gentamicin-loaded titania nanotubes with various diameters. *Int J Nanomedicine*. 2014;**9**:1215-30.
41. Pulido L, Ghanem E, Joshi A, Purtill JJ, Parvizi J. Periprosthetic joint infection: the incidence, timing, and predisposing factors. *Clin Orthop Relat Res*. 2008;**466(7)**:1710-5.
42. Hanssen AD. Local antibiotic delivery vehicles in the treatment of musculoskeletal infection. *Clin Orthop Relat Res*. 2005;**437**:91-6.
43. Cheal EJ, Spector M, Hayes WC. Role of loads and prosthesis material properties on the mechanics of the proximal femur after total hip arthroplasty. *J Orthop Res*. 1992;**10(3)**:405-22.
44. Naher S, O'Callaghan, D. DB. A Powered Technique for Inserting Acetabular Cups. *Adv Mat Res*. 2012;**445**:1011-6.
45. Md Jani AM, Losic D, Voelcker NH. Nanoporous anodic aluminium oxide: Advances in surface engineering and emerging applications. *Prog Mater Sci*. 2013;**58(5)**:636-704.
46. Geetha M, Singh AK, Asokamani R, Gogia AK. Ti based biomaterials, the ultimate choice for orthopaedic implants – A review. *Prog Mater Sci*. 2009;**54(3)**:397-425.
47. Ayon AA, Cantu M, Chava K, Agrawal CM, Feldman MD, Johnson D, et al. Drug loading of nanoporous TiO₂ films. *Biomed Mater*. 2006;**1(4)**:L11-5.

48. Ketabchi A, Komm K, Miles-Rossouw M, Cassani DA, Variola F. Nanoporous titanium surfaces for sustained elution of proteins and antibiotics. *PLoS One*. 2014;**9(3)**:e92080.
49. Lopez T, Manjarrez J, Rembao D, Vinogradova E, Moreno A, Gonzalez RD. An implantable sol–gel derived titania–silica carrier system for the controlled release of anticonvulsants. *Mater Lett*. 2006;**60(23)**:2903-8.
50. Chang YH, Tai CL, Hsu HY, Hsieh PH, Lee MS, Ueng SW. Liquid antibiotics in bone cement: an effective way to improve the efficiency of antibiotic release in antibiotic loaded bone cement. *Bone Joint Res*. 2014;**3(8)**:246-51.
51. Kim BM, Qian S, Bau HH. Filling carbon nanotubes with particles. *Nano Lett*. 2005;**5(5)**:873-8.
52. Kolahi Z. C60 Encapsulation inside Nitrogen-Doped and Pristine Multi-walled Carbon Nanotubes (MWCNTs): Investigation of the Dynamics of Encapsulated C60s inside Thin-Walled MWCNTs. Umeå: Umeå University; 2013.
53. Siegel RA, Rathbone MJ. Overview of Controlled Release Mechanisms. In: Siepmann J, Siegel RA, Rathbone MJ, editors. *Fundamentals and Applications of Controlled Release Drug Delivery*. Part of the series *Advances in Delivery Science and Technology*. New York: Springer US; 2011, p. 19-43.
54. Hacking SA, Tanzer M, Harvey EJ, Krygier JJ, Bobyn JD. Relative contributions of chemistry and topography to the osseointegration of hydroxyapatite coatings. *Clin Orthop Relat Res*. 2002;**405**:24-38.
55. Zanello LP, Zhao B, Hu H, Haddon RC. Bone cell proliferation on carbon nanotubes. *Nano Lett*. 2006;**6(3)**:562-7.
56. Firkowska I, Giannona S, Rojas-Chapana JA, Luecke K, Brüstle O, Giersig M. Biocompatible Nanomaterials and Nanodevices Promising for Biomedical Applications. In:

Giersig M, Khomutov GB, editors. *Nanomaterials for Application in Medicine and Biology*. Dordrecht: Springer Netherlands; 2008, p. 1-15.

57. Pantanella F, Berlutti F, Passeri D, Sordi D, Frioni A, Natalizi T, et al. Quantitative Evaluation of Bacteria Adherent and in Biofilm on Single-Wall Carbon Nanotube-Coated Surfaces. *Interdiscip Perspect Infect Dis*. 2011;**2011**:9.

58. Cui HF, Vashist SK, Al-Rubeaan K, Luong JH, Sheu FS. Interfacing carbon nanotubes with living mammalian cells and cytotoxicity issues. *Chem Res Toxicol*. 2010;**23**(7):1131-47.

59. Firme CP, 3rd, Bandaru PR. Toxicity issues in the application of carbon nanotubes to biological systems. *Nanomedicine*. 2010;**6**(2):245-56.

60. Pondman KM, Sobik M, Nayak A, Tsolaki AG, Jakel A, Flahaut E, et al. Complement activation by carbon nanotubes and its influence on the phagocytosis and cytokine response by macrophages. *Nanomedicine*. 2014;**10**(6):1287-99.

61. Bhattacharya K, Mukherjee SP, Gallud A, Burkert SC, Bistarelli S, Bellucci S, et al. Biological interactions of carbon-based nanomaterials: From coronation to degradation. *Nanomedicine*. 2016;**12**(2):333-51.

FIGURE LEGENDS

Figure 1: Scanning electron microscopy images of multi-walled carbon nanotube (MWCNT) coating on titanium alloy discs. A) shows a low magnification image of the rough titanium alloy disc covered homogenously with the MWCNT coating. B) shows a higher magnification of the area encircled in A) with a red square. The individual dots are the MWCNTs in top view and their size ranges from about 10-200 nm. (C) shows the same area in 45° tilt perspective and the corresponding projection of the MWCNTs into this image plane. The length of the MWCNTs is approximately 700 nm where few individual MWCNTs are longer. The darker shadows on the tips of the MWCNTs correspond to the encapsulated nickel particles from which the MWCNTs grew.

Figure 2: Antibacterial properties of Rifampicin-pretreated titanium alloy discs with or without MWCNT-coating or AB impregnation. A) *S. epidermidis* biofilms were grown on TiAl6V4 titanium alloy discs (n=3) for 24h after AB diffusion time periods of 1d to 10d. Next, biofilms were suspended, diluted and plated onto agar in triplicate. CFU were counted and mean values, SD and p-values were calculated. Bars are shown as mean values \pm SD. *** $p \leq 0.001$, n.s. = not significant. B) *S. epidermidis* suspensions from detached 24h-biofilms were measured for OD₄₉₀ absorbance in triplicate. Bars represent mean values \pm SD. * $p = 0.04$, ** $p < 0.01$, n.s. = not significant. C) Inhibition zones were determined by placing discs onto agar plates invertedly in triplicate. Horizontal bars are shown as mean values \pm SD. *** $p \leq 0.001$. All data represent the mean \pm SD of three independent experiments.